Universidade de Aveiro Departamento de Biologia
2016

Núria Tatiana Caeiro Lopes dos Inocentes

Lifetime Fitness Costs of Combined Effects of Fluoxetine and Increased Unpredictability in Temperature

Custos de *Fitness* ao Longo da Vida dos Efeitos Combinados de Fluoxetina e Imprevisibilidade na Temperatura

DECLARAÇÃO

Declaro que este relatório é integralmente da minha autoria, estando devidamente referenciadas as fontes e obras consultadas, bem como identificadas de modo claro as citações dessas obras. Não contém, por isso, qualquer tipo de plágio quer de textos publicados, qualquer que seja o meio dessa publicação, incluindo meios eletrónicos, quer de trabalhos académicos.

Universidade de Aveiro Departamento de Biologia
2016

Núria Tatiana Caeiro Lopes dos Inocentes

Lifetime Fitness Costs of Combined Effects of Fluoxetine and Increased Unpredictability in Temperature

Custos de *Fitness* ao Longo da Vida dos Efeitos Combinados de Fluoxetina e Imprevisibilidade na Temperatura

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha, realizada sob a orientação científica do Doutor Miguel Maria Borges da Costa Guint Barbosa, Professor Auxiliar Convidado do Departamento de Biologia da Universidade de Aveiro.

o júri

presidente	Doutora Ana Isabel Lillebø Batista Investigadora Principal do CESAM e Departamento de Biologia da Universidade de Aveiro
arguente	Doutor Marcos Rubal Garcia Investigador Pós-Doc do Centro de Investigação Marinha e Ambiental da Faculdade de Ciências da Universidade do Porto
orientador	Prof. Doutor Miguel Maria Borges da Costa Guint Barbosa Professor Auxiliar Convidado do Departamento de Biologia da Universidade de Aveiro

agradecimentos

Gostaria de agradecer em primeiro lugar ao Doutor Miguel Barbosa, por ter entrado nesta aventura comigo e por me ter dado um rumo no mundo académico, juntamente com a sabedoria (e simpatia!) necessária.

Ao Doutor Miguel Oliveira pelo apoio e por se ter mostrado sempre disponível, e também ao Doutor Victor Galhano pelos comentários ao longo do trabalho.

A todos os colegas do applEE que de certa forma me ajudaram sempre com um sorriso nos lábios, em especial ao técnico Abel Ferreira, a quem inúmeras vezes fui pedir ajuda quando me perdia no meio de tanta nova informação.

Ao Ricardo, obrigada por toda a ajuda durante estes anos, pelas manhãs, tardes, noites e fins de semana passados no laboratório a contar dáfnias, pelo apoio incondicional e pelos incontáveis “parece-me bem”!

Finalmente, aos meus pais, Cristina e Armando, sem os quais nada na minha vida académica (e na vida em geral!) seria possível. Obrigada por acreditarem em mim, por me ensinarem que nada na vida aparece sem trabalho, e que com determinação chego aos meus objetivos, por mais complicado que tudo possa parecer.

The flower that blooms in adversity is the most rare and beautiful of all.

palavras-chave

alterações climáticas, fluoxetina, efeitos combinados, fitness, história de vida, *Daphnia magna*

resumo

A temperatura é uma variável importante no controlo e modelação de todos os processos celulares, biológicos e fisiológicos de todos os organismos do Reino Animal. Por conseguinte, espera-se que alterações na temperatura ótima tenham um efeito significativo na reprodução, crescimento e desenvolvimento e, como tal, sobre o *fitness* do indivíduo. Há cada vez mais provas de que o stress causado por mudanças na temperatura impõe custos de *carry-over*, o que dificulta a capacidade dos organismos de lidar com outros stressores ambientais. É amplamente aceite que o ambiente está a mudar a um ritmo sem precedentes, com modelos de alterações globais antecipando um aumento na temperatura média. Mais preocupante, há fortes evidências de que a temperatura também se está a tornar imprevisível. A poluição antropogénica é um vetor de stress comum em sistemas aquáticos com um impacto direto sobre o *fitness* individual. A fluoxetina é um poluente vulgarmente encontrado em estações de tratamento de águas residuais. Inúmeros estudos reportaram custos de *fitness* relacionados com altas concentrações de fluoxetina. No entanto, e apesar da urgência do conhecimento da adaptação das espécies à mudança global, os efeitos combinados de múltiplos stressores ambientais permanecem pouco explorados. Além disso, até à data o conhecimento dos efeitos combinados de múltiplos stressores estão limitados a estudos de apenas uma geração, negligenciando os efeitos cruciais a longo prazo de *fitness*, falhando, portanto, em potencialmente detetar respostas adaptativas. Aqui, utilizando uma nova abordagem, os efeitos no *fitness* da exposição à fluoxetina ao longo de três gerações completas foram examinados, bem como os efeitos combinados do aumento da exposição e da imprevisibilidade da temperatura após a terceira geração. A pulga de água *Daphnia magna* foi exposta a três concentrações ambientalmente relevantes de fluoxetina: 0.000 µg/L (controlo), 0.012 µg/L (baixo) e 0.540 µg/L (alto). Na quarta geração, utilizando um design de factorização materna, cada linha maternal em fluoxetina foi exposta a três tratamentos de temperatura: uma média (20ºC), uma alta (25ºC) e uma imprevisível (15-25ºC). Como previsto, houve um custo adaptativo associado com a exposição crónica à fluoxetina, com menos neonatos produzidos durante a vida em baixa e alta concentração de fluoxetina do que nas produzidas na ausência (controlo). Inesperadamente, não houve um efeito na probabilidade de sobrevivência entre as três concentrações de fluoxetina. Após a introdução da temperatura, os indivíduos produzidos através da concentração alta e expostos

à temperatura imprevisível tinham uma redução no número de neonatos produzidos, em comparação com as posicionadas numa temperatura média e elevada. Nomeadamente, houve uma redução do número total de neonatos de 65 % e 75%, respetivamente. Novamente, não houve efeitos combinados sobre a sobrevivência. Modelos de eficiência alimentar e de alocação de energia podem fornecer explicações plausíveis para a falha na deteção de um efeito da fluoxetina por si só e combinados com a temperatura na sobrevivência. Em espécies ectotérmicas, o aumento do stresse reduz a eficiência alimentar, e menos energia obtida através da alimentação é priorizada a manutenção do corpo e crescimento, e só então para a reprodução. Este estudo fornece informação importante sobre uma nova abordagem para testes ecotoxicológicos, e uma nova compreensão sobre como os efeitos combinados das alterações globais e da poluição em sistemas aquáticos podem afetar organismos não-alvo.

keywords climate change, fluoxetine, combined effects, fitness, life history, *Daphnia magna*

abstract Temperature is a vital variable in controlling and shaping all cellular, biological and physiological processes of every organism of the animal kingdom. Consequently, changes in optimal temperature are expected to have a significant effect in reproduction, growth and development, hence on individual fitness. There is increasing evidence that stress caused by changes in temperature impose carry-over costs, which hinder the ability of organisms to deal with other environmental stressors. It is widely accepted that the environment is changing at an unprecedented pace, with global change models forecasting an increase in mean temperature. More worryingly, there is strong evidence that the temperature is also becoming unpredictable. Anthropogenic driven pollution is a vector of common stress in aquatic systems with a direct impact on individual fitness. Fluoxetine is a pollutant commonly found in wastewater treatment plants. Numerous studies have reported fitness costs related to high concentrations of fluoxetine. Yet, in spite of the recognized urgency of the understanding of species adaptation to global change, the combined effects of multiple environmental stressors remain little explored. Furthermore, to date our understanding of the combined effects of multiple stressors are limited to one-generation studies, neglecting crucial long-term effects of fitness, hence failing to potentially detect adaptive responses. Here, using a novel approach, the fitness effects of exposure to fluoxetine over three full generations were examined, as well as the combined effects with the exposure to increased unpredictability in temperature after the third generation. Namely, the water flea *Dapnia magna* was exposed to three environmentally relevant concentrations of fluoxetine: 0.000 µg/L (control), 0.012 µg/L (low) and 0.540 µg/L (high). In the fourth generation, using a maternal factorial design, each maternal fluoxetine line was exposed to three temperature treatments: an average (20ºC), a high (25ºC) and an unpredictable (15-25ºC). As predicted, there was a fitness cost associated with chronic exposure to fluoxetine, with less neonates produced during lifetime in low and high concentration of fluoxetine than those produced in absence (control). Unexpectedly, there was no effect in the probability of survival between the three fluoxetine concentrations. After the introduction of temperature, individuals produced via high concentration and exposed to unpredictable temperature had a reduction in the number of neonates produced in comparison with those allocated to an average and high temperature. Namely, there was a reduction of total number of neonates of 65% and 75%, respectively. Again, there were no combined effects on survival. Feeding efficiency and energy allocation models may provide with plausible explanations for the failure in detecting an effect of fluoxetine alone and combined with temperature in survival. In ectotherm species, increased stress reduces feeding efficiency, and the less energy obtained through feeding is prioritized to body maintenance and growth, and only then to reproduction. This study provides important information regarding a novel approach for ecotoxicological tests, and new insights in how the combined effects of global change and pollution in aquatic systems can affect non-target organisms.

Table of Contents

List of Figures and Tables

Introduction

Change is the only constant in natural systems. The environment is now changing at an unprecedented pace (IPCC, 2007), partially because of anthropogenic actions (Kerr, 2007). Disturbances are set to increase in intensity and frequency, but more worryingly they are also forecasted to become more unpredictable (Mora et al., 2013; Morice et al., 2012). Remarkably, there is little information on whether and how species adapt to keep up with environmental unpredictability (Barbosa et al., 2015; Gienapp et al., 2008; Hendry et al., 2008; Visser, 2008). It is now recognized that a large number of species can successfully adapt to changes in temperature by adjusting the timing of reproductive strategies in an adaptive manner (i.e. phenological responses) (Penuelas & Filella, 2001). Nonetheless, surprisingly, our understanding about species responses is limited to predictable changes in temperature, and less is known about how species respond when temperature variations become unpredictable as forecasted. This absence is startling, given the evidence that increased variation in temperature poses a greater threat to species than mean increase (Vasseur et al., 2014). Thus, investigating how species respond to unpredictable variation in temperature will give us a vital understanding of the ability of species to respond and adapt to global change.

Temperature is widely recognized as a determinant factor for growth, reproduction and development for ectotherm species (Cossins & Bowler, 1987; Brett, 1969). Due to thermoregulation and metabolic costs, a change in temperature is likely to affect the amount of energy available for reproduction and growth (Angilletta et al., 2004). Accordingly, an increased unpredictability in temperature will most likely cause physiological stress in organisms and affect individual energy balance, since it may directly affect its metabolic rate (Barbosa et al., 2014). Because the amount of available energy is often limited, an increased expenditure due to stress caused by increased unpredictability in temperature is likely to limit the ability of individuals to respond to additional non-environmental stressors.

Rivers and other aquatic watercourses are particularly vulnerable to climate change (IPCC, 2007). Additionally, these ecosystems are constantly receiving contaminants from

human-related activities. Thus, the effect of increased unpredictability in temperature caused by global change in combination with increased pollution is likely to impose an additional risk for species to adapt (Nougué et al., 2016; Zhang et al., 2016). It has been shown that some contaminants, even at low concentrations, have a negative effect on exposed organisms (e.g. nervous and endocrine system) (Lazzara et al., 2012). Fluoxetine is a human pharmaceutical and a highly prescribed selective serotonin reuptake inhibitor (SSRI), a contaminant commonly found in most aquatic ecosystems (Brooks et al., 2003a). It is released directly to the environment after passing through wastewater treatment plants (Kolpin et al., 2002), since fluoxetine is mainly excreted in human urine with less than 10% excreted unchanged (Vaswani et al., 2003). Studies have shown that fluoxetine hinders significantly the fitness of the exposed organisms by affecting vital functions such as reproduction and feeding (Weinberger & Klaper, 2014; Foster et al., 2010). For example, exposure to fluoxetine has been reported to have a negative effect on growth rate and lifetime reproductive success in the Japanese medaka (*Oryzias latipes*) (Foran et al., 2004) and in the water flea (*Daphnia magna*) (Luna et al., 2015; Flaherty & Dodson, 2005; Brooks et al., 2003b). It is believed that fluoxetine affects individuals' fitness because it acts directly on the hormonal and neuronal pathways, not only influencing food intake and assimilation, but also sexual behavior (Fent et al., 2006).

Despite being widely recognized that climate-related hazards exacerbate the effects of other non-naturally occurring stressors, hindering the ability to successfully respond to additional stressors (Harley et al., 2006), to date the general understanding of this relationship is limited. In this study this gap is addressed, and the hypothesis that fitness costs of increased unpredictability in temperature would be greater in individuals chronically exposed to greater concentration of fluoxetine than in individuals chronically exposed to lower concentrations is tested. This hypothesis was tested by quantifying lifetime reproductive success and probability of survival in individuals of *Daphnia magna* across three full generation exposed to different concentrations of fluoxetine and one generation in which each maternal treatment was allocated to a different temperature treatment.

The unique biological and ecological features of Daphnia make them an important

and commonly used model in ecological, evolutionary and toxicology studies (Ebert, 2005). Namely, daphnia have short generation times and large brood sizes. Furthermore, daphnia reproduces through cyclical parthenogenesis – which nullifies the possibility of genetic variation, as every descendent is a clone of the mother (Hebert & Ward, 1972). Importantly, daphnia are amendable to laboratory manipulation of temperature, with fitness responses to changes in optimal temperature easily observed (Barbosa et al., 2015, 2014). These are crucial prerequisites when tracking variation in long-term fitness. Daphnia are dominant organisms in ephemeral habitats who thrive in unpredictable environments, as they are able to maximize fitness in unpredictable conditions (Barbosa et al., 2015). They are also both primary consumers of phytoplankton and food source to secondary consumers. As a result of this role, any changes in fitness in daphnia caused by fluctuations in environmental conditions are thus likely to have repercussions in the entire ecosystem. Because of the unique biological characteristics, their responsiveness to laboratory experimentation and direct response to changes in temperature shown, allied to their vital role in ecosystem balance, daphnia are strong candidates to test the long-term fitness effects of chronic exposure to fluoxetine and increased unpredictability in temperature.

The pharmaceutical toxicity to *Daphnia magna* depends on the timing and duration of the exposure (Flaherty & Dodson 2005). It is important to perform chronic life-cycle tests – because pharmaceuticals – namely fluoxetine – are being continuously released into the environment and evidence for its long-term ecological effects is accumulating (Brausch et al., 2012; Brooks et al., 2003a; Brooks et al., 2003b). The sublethal effects of fluoxetine on reproduction are most likely in aquatic invertebrates (Péry et al., 2008). Studies on the effect of fluoxetine on the reproductive biology of *D. magna* indicate that concentrations of fluoxetine greater than 36 µg/L significantly increased fecundity (Flaherty & Dodson, 2005). However, for other species, ranges of fluoxetine concentrations between 20 µg/L to 340 µg/L have been reported to elicit spawning in zebra mussels (*Dreissena polymorpha*) (Lazzara et al., 2012; Fong, 1998). By contrast, chronic exposure to much smaller concentrations of fluoxetine (0.012-0.099 µg/L) cause developmental delays and decreased growth in tadpoles of northern leopard frogs (*Rana pipiens*) (Foster et al., 2010).

Daphnia are ectotherm organisms, which means they are unable to internally control body temperature. Because of this, any change to outside (environmental) temperature is followed by a direct effect on all biological and physiological processes. Multiple studies have reported that time between broods in *Daphnia magna* decreases as growth rate increases in individuals reared at constant temperatures, in comparison with individuals reared at unpredictable temperatures (Barbosa et al., 2014). This result is consistent with the knowledge that fitness is affected negatively when temperature is above optimal conditions (Martin & Huey, 2008).

In order to investigate the combined long term fitness effects of chronic exposure to fluoxetine and increased unpredictability in temperature, a multigenerational test was conducted in which four consecutive generations of *Daphnia magna* were exposed to control, low and high concentrations of fluoxetine during their entire life. In the fourth generation, organisms from each fluoxetine treatment were exposed to three temperatures treatments (average, high and variable). During this time the total number of neonates produced during the entire life and time of survival rate were quantified.

Material and Methods

The tested hypothesis was that fitness costs of increased unpredictability in temperature would be greater among individuals chronically exposed to fluoxetine than among individuals with lower levels of exposure to fluoxetine. The combined effect of early exposure to fluoxetine and increased variability in temperature on lifetime reproductive success was examined on the 4^{th} generation of individuals that had been exposed to different concentrations of fluoxetine for three generations.

All F0 individuals used in this study were 4^{th} brood neonates generated from *D. magna* clone F (Baird et al., 1991) raised at the constant temperature of 20° C in a 16:8 hour light:dark photoperiod in ASTM (American Society for Testing Materials) and fed with green algae *Raphidocelis subcapitata*, at a concentration of 3.0×10^5 cells mL^{-1}. This clone was chosen due to its responsiveness to environmental stress detected in previous studies (Barbosa et al., 2015, 2014). Immediately after birth, 30 F0 individuals were randomly allocated to one of three fluoxetine concentration treatments, low (n=10, 0.012 µg/L), high (n=10, 0.540 µg/L) and a control (n=10, 0.000 µg/L) individually, 10 per treatment, in 50 mL glass beakers. Low and high fluoxetine concentrations were chosen accordingly to maximum values found in surface waters and municipal effluents, respectively (Kolpin et al., 2002; Weston et al., 2001). F0s were checked daily for neonates (F1). After the birth of brood number four, the individuals (F1) were collected and allocated in the same concentration treatment as their mother (i.e. these ones were used as F1). Forty F1 neonates per concentration treatment were used. Each F1 was individually checked for neonates every day. When the brood number four of F1 was produced (i.e. F2), these individuals were then allocated to the same concentration treatment of their parental line. Again, 40 F2 neonates per fluoxetine concentration were allocated. The procedure was repeated, and F3 neonates were checked for every day. Yet again, at brood number four, 40 F3 were allocated in each fluoxetine concentration treatment. At brood number four of F3 (i.e. F4), the temperature treatment was added. A total of 40 individuals per concentration treatment were randomly allocated to one of three temperature treatments designated as average (20°C), high (25°C) and variable (15 to 25°C). The temperature in the

variable treatment varied in an unpredictable way. However, to avoid unrealistic temperatures, the temperature varied according to 2 sub-groups - 00:00 to 08:00/ 18:00 to 24:00 (dawn-morning/late afternoon) and from 08:00 to 18:00 (morning and afternoon). In the dawn-morning/late afternoon sub-group, the temperature fluctuated unpredictably between 15°C and 20°C. In the morning and afternoon sub-group, it varied between 20°C and 25°C (Table 1). For all individuals across all generations, the medium and food were renewed every other day. All individuals (F0, F1, F2, F3, F4) remained in their conditions until dead.

	Time of the Day (h)					
Day	**0-4**	**4-8**	**8-12**	**12-16**	**16-20**	**20-24**
Monday	14~15ºC	15~16ºC	16~22ºC	22~26ºC	26~20ºC	20~14ºC
Tuesday	14~15ºC	15~16ºC	16~22ºC	22~20ºC	20~18ºC	18~14ºC
Wednesday	14~15ºC	15~17ºC	17~26ºC	26ºC	26~24ºC	24~20ºC
Thursday	20~16ºC	16~15ºC	15~20ºC	20~22ºC	22~26ºC	26~14ºC
Friday	14~16ºC	16~17ºC	17~20ºC	20ºC	20~17ºC	17~15ºC
Saturday	15~16ºC	16ºC	16~20ºC	20~26ºC	26~22ºC	22~15ºC
Sunday	15~16ºC	16~17ºC	17~19ºC	19~20ºC	20~26ºC	26~14ºC

Table 1 – Temperature variation over 24 hours on the F4 treatment. This cycle was repeated weekly.

Statistics

The fitness effect of increased variation in temperature on individuals previously exposed to different concentrations of fluoxetine was analyzed using a Generalized Linear Model (GLM). The fixed effect structure of the model included concentration, temperature and time. Diagnostics plots indicate that total variation of number of neonates produced during lifetime departs from a Gaussian distribution. For this reason, in both models, the effect of concentration treatment and temperature treatment on lifetime reproductive success were modeled using a Poisson distribution.

For each model, all factors were tested for the minimal adequate model using Akaike's Information Criterion (Burnham & Anderson, 2002). Specifically, ΔAIC, the difference between the AIC of each model and that of the estimated best model (the model with the lowest AIC) was calculated. Akaike weights, which are estimates of the probability that each model is the best in the model set was also calculated to assess uncertainty about the best model (reflected in multiple models having similar Akaike weights). Post-hoc multiple pairwise comparisons between groups using the multcomp package (Hothorn et al., 2008) were used to test which group means were different after a significant hypothesis test.

To examine the scale of the combined effects of fluoxetine concentration and increased unpredictability of temperature on lifetime reproductive success, a log ratio test (LRT) was used. Namely, the logarithmic of the ratio between the number of neonates produced by each concentration treatment along generations and the number of neonates produced by each temperature treatment was calculated.

Finally, the effect of increased variation in temperature on individuals exposed to fluoxetine on the probability of survival was analyzed using a Cox-proportional hazard model.

All *p*-values were considered significant after Bonferroni correction and all analyses were performed in R (Team, 2016), using packages nlme (Pinheiro & Bates, 2000) and coxme (Therneau, 2015).

Results

There were more neonates produced during lifetime under no fluoxetine (i.e. control) than in the other two fluoxetine concentrations (Table 2). However, the results also showed a decrease in the number of neonates along the three generations for all fluoxetine concentrations (Figure 1). Nevertheless, the scale of the decrease was greater in individuals exposed to the high concentration of fluoxetine than in individuals exposed to the low concentration and control. Exposure to the high concentration of fluoxetine induced a reduction of 55% on total number of neonates produced by the 3rd generation, whereas in the low and control concentration the reduction was of 43% and 41%, respectively.

There was a significant effect of temperature and fluoxetine on the number of neonates produced after the 3rd generation, but this effect differed along time (Figure 1, Tables 2 and 3). Surprisingly, for all fluoxetine concentrations there were on average more neonates produced under variable temperature than on the two other temperature treatments (Figure 2, Table 2). Importantly, neonates produced via mothers that were exposed to low and control concentrations of fluoxetine mostly drove this increase in neonate numbers in the variable temperature treatment. In fact, there was a dramatic fitness cost associated with the maternal line high fluoxetine - variable temperature, of 65% regarding the maternal line control fluoxetine - variable temperature, and of 75% in comparison to the maternal line low fluoxetine - variable temperature (Figure 2, Table 2).

Survival Curve

There was no significant effect of concentration of fluoxetine on the probability of survival (Wald Test=0.21, p=0.901, Figure 3, Table 4 and 5). In the control treatment, it took on average 72 days to observe a reduction in 50% of the number of neonates. In low and high concentrations of fluoxetine these were achieved faster, in 60 and 68 days respectively (Figure 3A).

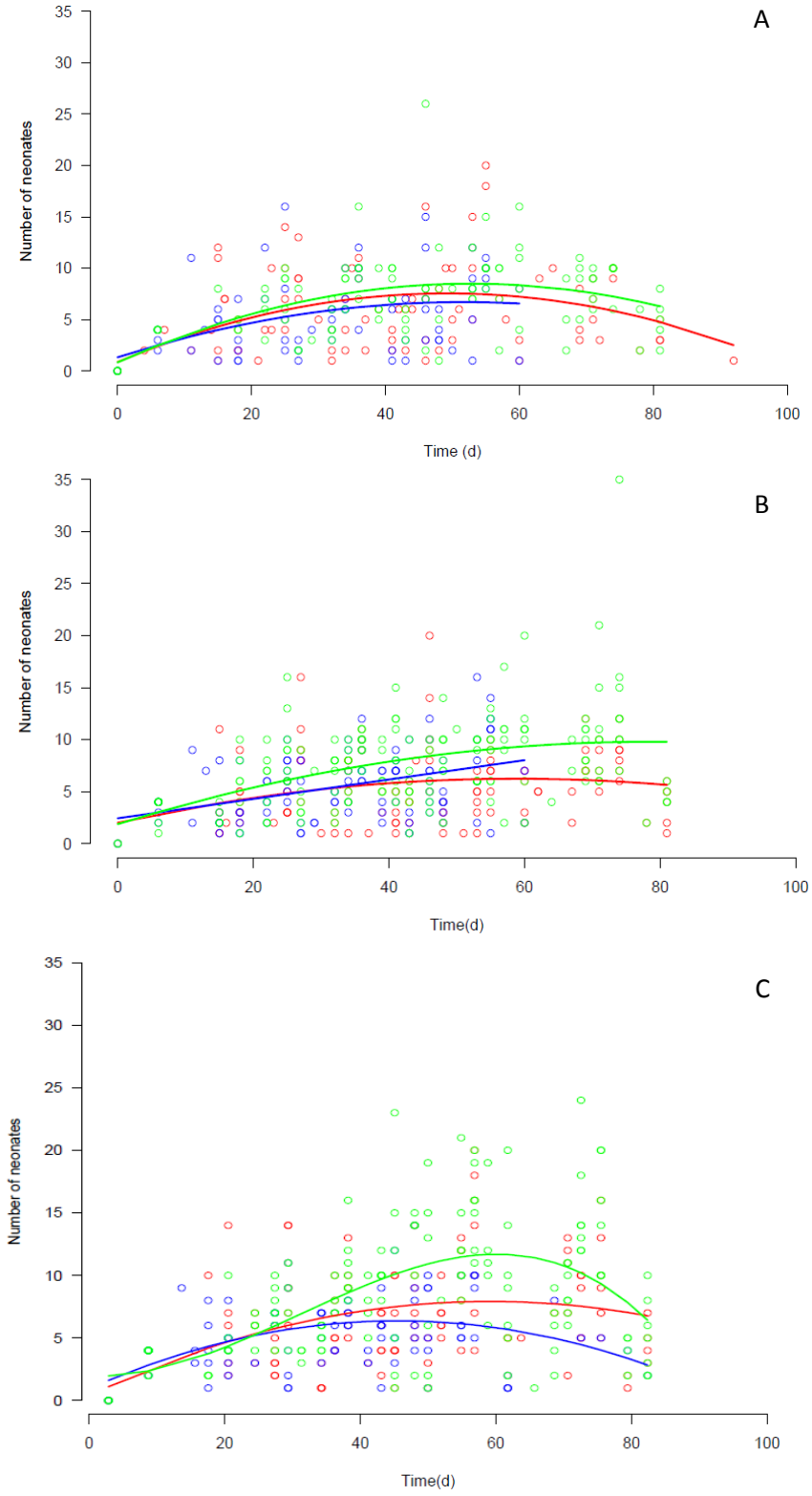

Figure 1 – Life trajectories for individuals exposed to control (A), low (B) and high (C) concentrations of fluoxetine and allocated to a context of average temperature (blue), high temperature (red) or variable temperature (green). Lines represent best-fitted model determined by model selection using Akaike weights.

The introduction of the new variable temperature to organisms from each of the fluoxetine concentrations produced surprising results. Individuals reared under control concentration and subsequently allocated to a high temperature treatment showed a 3-fold increase in the probability of dying in comparison to individuals reared under average temperature (Figure 3, Table 4).

Generation	Fluoxetine Treatment	Total Number of Neonates		
1 to 3	Control	13540 (18.1 ± 14.3)		
	Low	9874 (17.1 ±14)		
	High	12696 (19.2 ± 16.3)		
		Temperature Condition		
		Average	High	Variable
4	Control	821 (20.5 ± 13.9)	551 (13.7±9.7)	1273 (31.8±21.6)
	Low	1209 (17.5±13.6)	992 (14.3±9.6)	1803 (26.1±19.4)
	High	136 (12.3±10.3)	128 (11.6±9.8)	444 (40.3±5.2)

Table 2 – Total number of neonates produced during lifetime via fluoxetine concentration and temperature treatment. Means and standard deviations are given (mean ± SD).

Model AIC	Explanatory variable	df	Sum Sq	F value	p-value
Reactive distance AIC - 63.4	Treatment	2	170	5.28	0.005
	Temperature	2	1120	34.9	<0.001
	Time	1	282	17.5	<0.001
	Treatment: Temperature	4	108	1.68	0.150

Table 3 – Generalized linear models for testing the effect of exposure to fluoxetine and temperature on number of neonates produced over lifetime. Model selection was performed using Akaike's Information Criterion (AIC). Only maximal adequate model is shown. The models with the lowest AIC was selected as being the minimum adequate model.

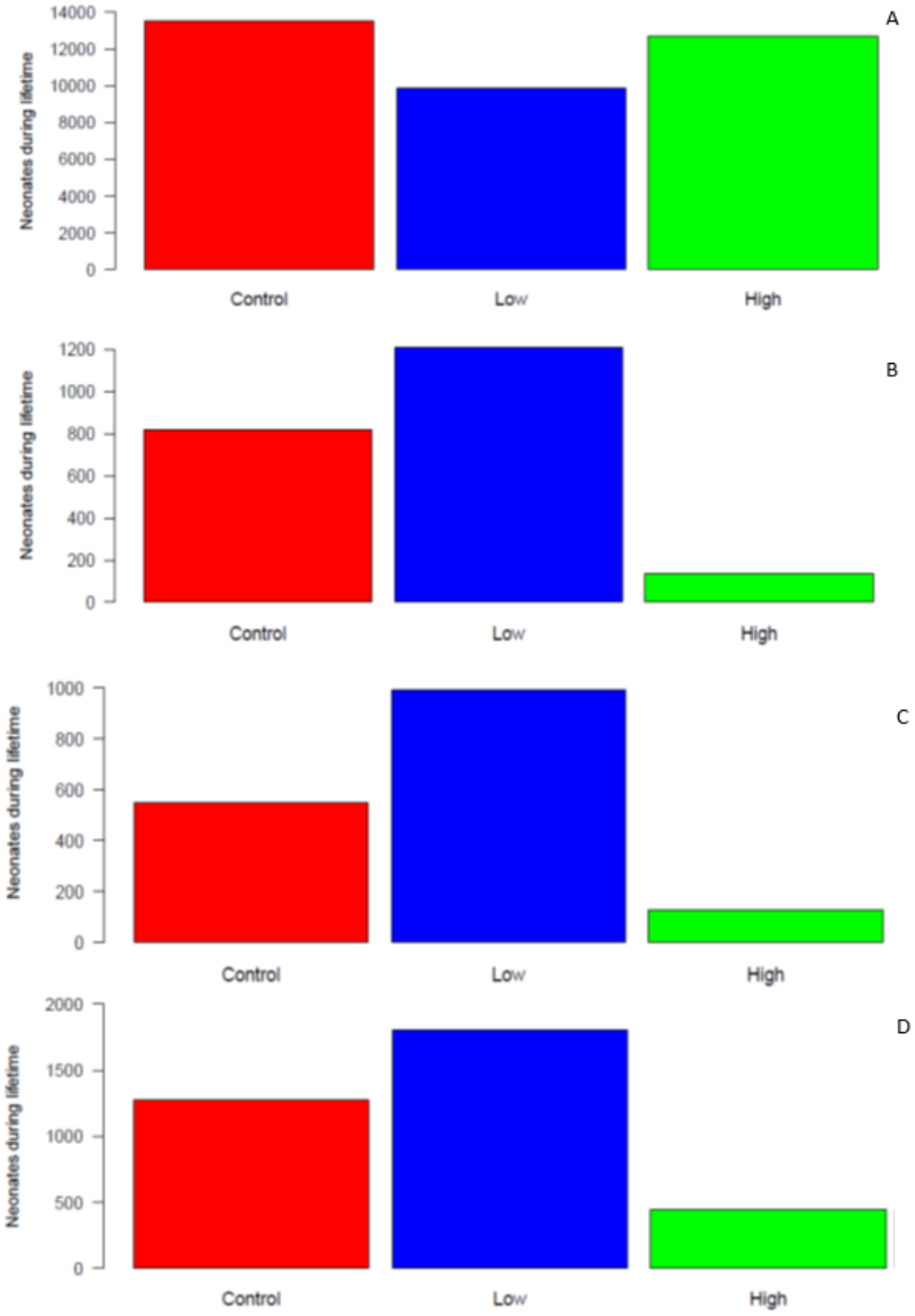

Figure 2 – Total number of neonates produced during lifetime after being exposed during three full generations to control, low, and high concentration of fluoxetine (A), and subsequently on the fourth generation, being exposed to average (B), high (C), and variable (D) temperature.

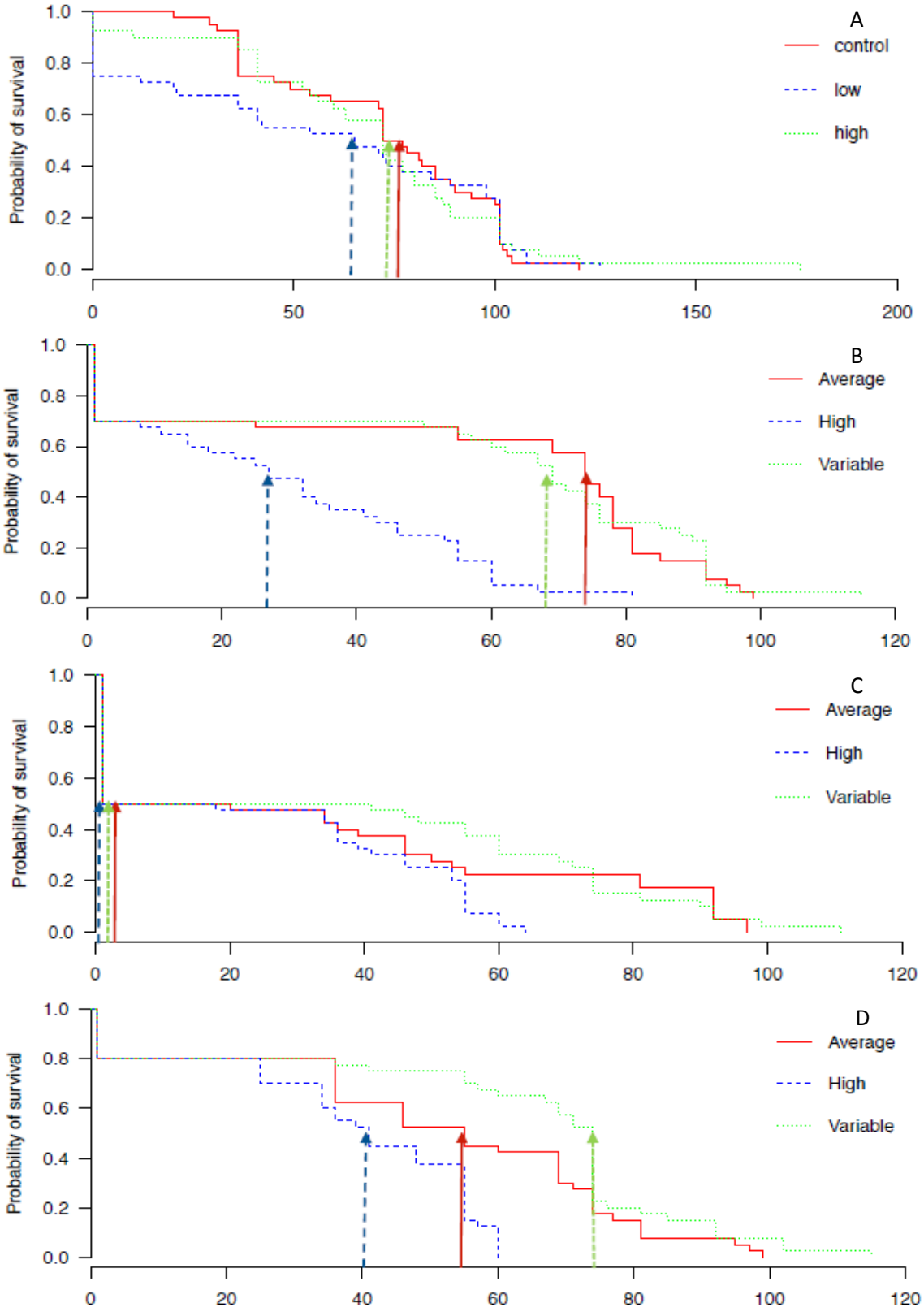

Figure 3 – Survival curves for individuals exposed to control, low and high concentration of fluoxetine during three generations (A). Survival curves for individuals produced in each of the three temperature treatments via mothers reared in control (B), low (C) or high (D) concentration of fluoxetine. Vertical lines represent the mean time needed for median survival.

Generation	Fluoxetine		coef	exp(coef)	z	Pr(>|z|)
1 to 3	Low		0.086	1.090	0.384	0.701
	High		-0.004	0.995	-0.020	0.984
4	Fluoxetine	Temperature	coef	exp(coef)	z	Pr(>|z|)
	Control	High	1.216	3.374	4.723	<0.001
		Variable	-0.013	0.986	0.226	0.953
	Low	High	0.445	1.560	1.876	0.060
		Variable	-0.123	0.884	-0.539	0.589
	High	High	1.020	2.773	3.967	<0.001
		Variable	-0.440	0.643	-1.921	0.054

Table 4 – Results from the Cox proportional hazards survival model. Survival probability is compared to the baseline (i.e. to the control treatment). Values of expected coefficient higher than 1 indicate a greater probability of survival in comparison to the baseline, whereas values smaller than one represent lower probability. Significant results were obtained using z test.

Previous exposure to low concentration of fluoxetine surprisingly did not add any cost of probability of survival in the three temperature treatments (Figure 3, Tables 4 and 5). Previous exposure to high concentration of fluoxetine significantly increased the likelihood of dying in individuals reared in both high and variable temperatures; namely, there was a 2-fold increase of dying in individuals in the high temperature treatment in comparison with those in the average temperature treatment (Figure 3, Tables 4 and 5).

Generation	Fluoxetine Treatment	Time of Survival (d)		
1 to 3	Control	71.6 (±27.6)		
	Low	56.2 (±42.6)		
	High	68.3 (±35.0)		
		Temperature Condition		
		Average	High	Variable
4	Control	54.3 (±37.4)	28.2 (±23.9)	55.0 (±37.9)
	Low	32.3 (±36.7)	23.7 (±24.4)	36.1 (±37.9)
	High	49.8 (±30.2)	36.8 (±21.0)	59.6 (±33.0)

Table 5 – Mean number of days (± SD) until death for generations one to three, in which individuals were exposed to a control, low, and high concentration of fluoxetine, and for generation 4, in which each fluoxetine treatment was exposed to an average, high, or variable temperature.

Discussion

In this study the hypothesis that the fitness costs are greater to individuals exposed to multiple sources of environmental stress than to individuals exposed to single stressors was tested. Using a multi-generational approach, it was observed that individuals chronically exposed to a high concentration of fluoxetine and allocated to a variable temperature treatment produced fewer neonates during their lifetime than in the other treatments. There was a 65% reduction in the total number of neonates produced during lifetime in comparison to those descended via control treatment and a 75% reduction in those produced via low concentration of fluoxetine. These conditions had, nevertheless, a negligible effect on lifespan. Individuals reared under high concentration of fluoxetine and exposed to unpredictable temperature survived as long as those in the other temperature treatments. By contrast, individuals reared under low concentration of fluoxetine showed the greatest decrease in survival in all temperature treatments, their survival probability dropping to 50% almost immediately. All in all, there was a considerable fitness cost for *D. magna* when exposed to the conditions of high concentration of fluoxetine and variable temperature – a scenario forecasted to happen under future global warming. The results of my study reinforce suggestions that individuals already stressed are at higher risk under increased unpredictability in temperature. Recent global change models forecast an increase in both the mean and variance in temperature. This means that populations and species that are currently under stress due to other environmental factors are more likely to be affected negatively by an increase in the unpredictability of temperature.

The fact that a reduction in fitness was only observed on the number of neonates generated but not on lifespan may be explained by energy allocation models. The Dynamic Energy Budget (DEB) is an individual-based theoretical framework design to model the rate at which assimilated resources are transformed into energy and allocated to growth, reproduction and maintenance as a function of the organism state and environmental conditions (Kooijman, 1986). One of the main assumptions of the model is that for ectotherm organisms all energy produced via feeding is primarily allocated to growth and maintenance and the remaining energy to reproduction (1-K rule) (Metcalf, 2016; Kooijman, 1986). For many different species an increase in stress leads to a reduction in feeding time (Ferrier-Pagès et al., 2010; Pincebourde et al., 2008; Hansen, 2004; Donkin et al., 1989). A reduction in feeding time or efficiency is expected to have been greater among individuals produced via the maternal line high concentration of fluoxetine - variable temperature. It is possible that the failure to detect an effect on the probability of survival under the high fluoxetine - variable temperature is that all the energy collected from the algae was allocated for maintenance and growth, causing a deficit in energy for reproduction. This seemingly efficient use of energy would allow organisms to survive as long as the ones in less pernicious conditions, although with the disadvantage of reduction in the number of neonates produced.

Examining the combined effects of fluoxetine and increased unpredictability in temperature provides a vital knowledge on if and how species would be able to adapt under the upcoming scenarios of global change. The results obtained in the three generations in which the only stressful vector was fluoxetine showed that this stress alone has a negligible effect on overall fitness. While individuals allocated to the control treatment (no fluoxetine) produced 6% and 27% more neonates than those produced by individuals in the high and low fluoxetine treatments, respectively, this difference was not statistically significant. In addition, the trend in reduction of the number of neonates along generations was similar for the three fluoxetine treatments. Finally, exposure to fluoxetine did not decrease the probability of survival. These results combined are a strong indication that fluoxetine alone has a minimal impact on lifetime fitness of daphnia. The effect of temperature can be estimated from the treatments where there was no fluoxetine involved (control). Overall there were more neonates produced under the variable temperature treatment than produced by the other two temperature treatments. Notably, this great number was mostly driven by neonates produced via low fluoxetine - variable temperature and control - variable temperature lines. By contrast, the high fluoxetine - variable temperature line had the lowest number of neonates produced of all. This was a reduction of 65% and 75% in comparison with the other two fluoxetine concentrations and allocated to the variable temperature treatment. The results also showed that individuals allocated to the high temperature treatment suffered a decrease in their probability of survival. Individuals allocated to the variable temperature treatment were also observed to live longer than any. These results demonstrate a clear impact of temperature in the fitness of daphnia.

Neonate number increase due to fluoxetine exposure is an expectable result in *Daphnia magna*, as it has been shown before in previous studies (Luna et al., 2015; Flaherty & Dodson, 2005; Brooks et al., 2003b). Albeit the concentrations selected by Luna et al. (2015) were far greater than the ones used in this study, exposure to 100 µg/L of fluoxetine for 40 days resulted in increased mortality in *Daphnia magna*, as well as increased neonate production and decreased population growth.

On the other hand, individuals allocated in low concentrations of fluoxetine showed a steep survival curve, while producing the biggest number of neonates in all temperatures. This implies that they invest all their energy on the next generation of neonates at the expense of their own survival, an example of bet-hedging: the mother’s fitness is maximized while her lifetime is decreased in response to unpredictable changes in the environment (Seger & Brockmann, 1987). It also correlates with Martin and Huey's (2008) evidence that daphnia face sharper declines in fitness when temperature is above optimal conditions, which in this case is aggravated by temperatures below optimal conditions as well.

These results are consistent with Barbosa et al. (2014) study that demonstrated that increased variability in temperature causes organisms to face greater difficulties in adapting. Several studies have also shown that species are more vulnerable to

unpredictable temperatures than changes in mean temperature (Rahmstorf & Coumou, 2011; Hawkins, 1995). This corroborates the presented results, as survivability is lower in individuals allocated in the unpredictable temperature treatments.

Conclusion

Overall, this study contributes for a better understanding of how organisms respond to a real situation and provided the needed scientific knowledge of two topics of concern: the pollution of aquatic systems through the presence of pharmaceuticals and the effects of climate change. The adopted approach, using a multi-generational test was expected to deliver much more representative data than ecotoxicological tests of 21 days. Examining the combined effects of two important ecological stressors along several generations, provided by a stronger test to accurately describe life trajectories, permits a more complete understanding of if and how organisms can adapt to global change under natural conditions. Using a generational approach in which maternal lines are subset into a factorial design also allowed to partition the variance in fitness between the direct effects (fluoxetine), indirect effects (temperature) and combined effects (fluoxetine and temperature). Studies like the one here presented are, therefore, more representative and environmentally relevant, which is an important scientific progress in methodology.

Bibliography

Angilletta, M. J., Steury, T. D., & Sears, M. W. (2004). Temperature , Growth Rate , and Body Size in Ectotherms : Fitting Pieces of a Life-History Puzzle. *Integrative and Comparative Biology*, *44*, 498–509. http://doi.org/10.1093/icb/44.6.498

Baird, D. J., Barber, I., Bradley, M., Soares, A. M. V. M., & Calow, P. (1991). A comparative study of genotype sensitivity to acute toxic stress using clones of Daphnia magna straus. *Ecotoxicology and Environmental Safety*, *21*(3), 257–265. http://doi.org/10.1016/0147-6513(91)90064-V

Barbosa, M., Lopes, I., Venâncio, C., Janeiro, M. J., Morrisey, M. B., & Soares, A. M. V. M. (2015). Maternal response to environmental unpredictability. *Ecology and Evolution*. http://doi.org/10.1002/ece3.1723

Barbosa, M., Pestana, J., & Soares, A. M. V. M. (2014). Predation Life History Responses to Increased Temperature Variability. *PLOS One*, *9*(9). http://doi.org/10.1371/journal.pone.0107971

Brausch, J. M., Connors, K. A., Brooks, B. W., & Rand, G. M. (2012). Human Pharmaceuticals in the Aquatic Environment: A Review of Recent Toxicological Studies and Considerations for Toxicity Testing. *Reviews of Environmental Contamination and Toxicology*, *218*(January). http://doi.org/10.1007/978-1-4614-3137-4

Brett, J. R. (1969). Temperature and fish. *Chesapeake Science*, *10*(3), 275–276. http://doi.org/10.2307/1350466

Brooks, B. W., Foran, C. M., Richards, S. M., Weston, J., Turner, P. K., Stanley, J. K., … La Point, T. W. (2003). Aquatic ecotoxicology of fluoxetine. *Toxicology Letters*, *142*(3), 169–183. http://doi.org/10.1016/S0378-4274(03)00066-3

Brooks, B. W., Turner, P. K., Stanley, J. K., Weston, J. J., Glidewell, E. A., Foran, C. M., … Huggett, D. B. (2003). Waterborne and sediment toxicity of fluoxetine to select organisms. *Chemosphere*, *52*(1), 135–142. http://doi.org/10.1016/S0045-6535(03)00103-6

Burnham, K. P., & Anderson, D. R. (2002). *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach (Second Edition)*. *Model Selection and Multimodel Inference: A Practical Information-Theoretic Approach (Second Edition)*. New York: Springer. Retrieved from http://linkinghub.elsevier.com/retrieve/pii/S0304380003004526

Cossins, A. R., & Bowler, K. (1987). *Temperature Biology of Animals*. *Temperature Biology of Animals*. London & New York: Chapman and Hall.

Donkin, P., Widdows, J., Evans, S. V., Worrall, C. M., & Carr, M. (1989). Quantitative structure-activity relationships for the effect of hydrophobic organic chemicals on rate of feeding by mussels (Mytilus edulis). *Aquatic Toxicology*, *14*(3), 277–294. http://doi.org/10.1016/0166-445X(89)90021-0

Ebert, D. (2005). *Ecology, Epidemiology and Evolution of Parasitism in Daphnia*. *Evolution* (Vol. 3). Bethesda (MD): National Library of Medicine. http://doi.org/10.1108/02634501111102760

Fent, K., Weston, A. A., & Caminada, D. (2006). Ecotoxicology of human pharmaceuticals. *Aquatic Toxicology (Amsterdam, Netherlands)*, *76*(2), 122–159. http://doi.org/10.1016/j.aquatox.2005.09.009

Ferrier-Pagès, C., Rottier, C., Beraud, E., & Levy, O. (2010). Experimental assessment of the feeding effort of three scleractinian coral species during a thermal stress: Effect on the rates of photosynthesis. *Journal of Experimental Marine Biology and Ecology*, *390*(2), 118–124. http://doi.org/10.1016/j.jembe.2010.05.007

Flaherty, C. M., & Dodson, S. I. (2005). Effects of pharmaceuticals on Daphnia survival, growth, and reproduction. *Chemosphere*, *61*(2), 200–207. http://doi.org/10.1016/j.chemosphere.2005.02.016

Fong, P. P. (1998). Zebra Mussel Spawning Is Induced in Low Concentrations of Putative Serotonin Reuptake Inhibitors. *The Biological Bulletin*, *194*(2), 143–149. http://doi.org/10.2307/1543044

Foran, C. M., Weston, J., Slattery, M., Brooks, B. W., & Huggett, D. B. (2004). Reproductive

Assessment of Japanese Medaka (Oryzias latipes) Following a Four-Week Fluoxetine (SSRI) Exposure. *Archives of Environmental Contamination and Toxicology*, *46*(4), 511–517. http://doi.org/10.1007/s00244-003-3042-5

Foster, H. R., Burton, G. A., Basu, N., & Werner, E. E. (2010). Chronic exposure to fluoxetine (Prozac) causes developmental delays in Rana pipiens larvae. *Environmental Toxicology and Chemistry*, *29*(12), 2845–2850. http://doi.org/10.1002/etc.345

Gienapp, P., Teplitsky, C., Alho, J. S., Mills, J. A., & Merilä, J. (2008). Climate change and evolution: disentangling environmental and genetic responses. *Molecular Ecology*, *17*(1), 167–178. http://doi.org/10.1111/j.1365-294X.2007.03413.x

Hansen, P. J. (2004). Physiological and cellular adaptations of zebu cattle to thermal stress. *Animal Reproduction Science*, *82–83*, 349–360. http://doi.org/10.1016/j.anireprosci.2004.04.011

Harley, C. D. G., Hughes, A. R., Hultgren, K. M., Miner, B. G., Sorte, C. J. B., Thornber, C. S., … Williams, S. L. (2006). The impacts of climate change in coastal marine systems. *Ecology Letters*, *9*(2), 228–241. http://doi.org/10.1111/j.1461-0248.2005.00871.x

Hawkins, A. J. S. (1995). Effects of temperature change on ectotherm metabolism and evolution: Metabolic and physiological interrelations underlying the superiority of multi-locus heterozygotes in heterogeneous environments. *Journal of Thermal Biology*, *20*(1–2), 23–33. http://doi.org/10.1016/0306-4565(94)00023-C

Hebert, P. D. N., & Ward, R. D. (1972). Inheritance during parthenogenesis in Daphnia magna. *Genetics*, *71*(4), 639–642. http://doi.org/10.1002/jez.488

Hendry, A. P., Farrugia, T. J., & Kinnison, M. T. (2008). Human influences on rates of phenotypic change in wild animal populations. *Molecular Ecology*, *17*(1), 20–29. http://doi.org/10.1111/j.1365-294X.2007.03428.x

Hothorn, T., Bretz, F., & Westfall, P. (2008). Simultaneous Inference in General Parametric Models. *Biometrical Journal*, *50*(3), 346–363. Retrieved from http://cran.r-project.org

IPCC. (2007). *Climate Change 2007 Synthesis Report*. *Contribution of Working Groups I, II and III to the Fourth Assessment Reposrt of the Intergovernmental Panel on Climate Change*. Geneva, Switzerland. http://doi.org/10.1256/004316502320517344

Kerr, R. A. (2007). Global Warming Is Changing the World. *Science*, *316*, 188–190. http://doi.org/10.1126/science.316.5822.188

Kolpin, D. W., Furlong, E. T., Meyer, M. T., Thurman, E. M., Zaugg, S. D., Barber, L. B., & Buxton, H. T. (2002). Pharmaceuticals, Hormones and Other Organic Wastewater Contaminants in U.S. Streams, 1999-2000: A National Reconnaissance. *Environmental Science & Technology*, *36*(6), 1202–1211. http://doi.org/10.1021/es011055j

Kooijman, S. A. L. M. (1986). Population dynamics on the basis of budget. In J. A. J. Metz & O. Diekman (Eds.), *Lecture Notes in Biomathematics, Volume 86: The Dynamics of Physiologically Structured Populations* (pp. 266–297). Berlin Heidelberg GmbH: Springer-Verlag. http://doi.org/10.1007/978-3-662-13159-6

Lazzara, R., Blázquez, M., Porte, C., & Barata, C. (2012). Low environmental levels of fluoxetine induce spawning and changes in endogenous estradiol levels in the zebra mussel Dreissena polymorpha. *Aquatic Toxicology*, *106–107*, 123–130. http://doi.org/10.1016/j.aquatox.2011.11.003

Luna, T. O., Plautz, S. C., & Salice, C. J. (2015). Chronic Effects of 17α-Ethinylestradiol, Fluoxetine, and the Mixture on Individual and Population-Level End Points in Daphnia magna. *Archives of Environmental Contamination and Toxicology*, *68*(4), 603–611. http://doi.org/10.1007/s00244-014-0119-2

Martin, T. L., & Huey, R. B. (2008). Why “Suboptimal” Is Optimal: Jensen’s Inequality and Ectotherm Thermal Preferences. *The American Naturalist*, *171*(3), E102–E118. http://doi.org/10.1086/527502

Metcalf, C. J. E. (2016). Invisible Trade-offs: Van Noordwijk and de Jong and Life-History Evolution. *American Naturalist*, *187*. http://doi.org/10.1086/685487

Mora, C., Frazier, A. G., Longman, R. J., Dacks, R. S., Walton, M. M., Tong, E. J., …

Giambelluca, T. W. (2013). The projected timing of climate departure from recent variability. *Nature*, *502*(7470), 183–196. http://doi.org/10.1038/nature12540

Morice, C. P., Kennedy, J. J., Rayner, N. A., & Jones, P. D. (2012). Quantifying uncertainties in global and regional temperature change using an ensemble of observational estimates: The HadCRUT4 data set. *Journal of Geophysical Research*, *117*(8). http://doi.org/10.1029/2011JD017187

Nougué, O., Svendsen, N., Jabbour-Zahab, R., Lenormand, T., & Chevin, L.-M. (2016). The ontogeny of tolerance curves: Habitat quality versus acclimation in a stressful environment. *Journal of Animal Ecology*, *85*, 1625–1635. http://doi.org/10.1111/1365-2656.12572

Penuelas, J., & Filella, I. (2001). Responses to a Warming World. *Science*, *294*(5543), 793–794. http://doi.org/10.1126/science.1066860

Péry, A. R. R., Gust, M., Vollat, B., Mons, R., Ramil, M., Fink, G., … Garric, J. (2008). Fluoxetine effects assessment on the life cycle of aquatic invertebrates. *Chemosphere*, *73*(3), 300–304. http://doi.org/10.1016/j.chemosphere.2008.06.029

Pincebourde, S., Sanford, E., & Helmuth, B. (2008). Body temperature during low tide alters the feeding performance of a top intertidal predator. *Limnology and Oceanography*, *53*(4), 1562–1573. http://doi.org/10.4319/lo.2008.53.4.1562

Pinheiro, J. C., & Bates, D. M. (2000). *Mixed-Effects Models in S and S-PLUS*. *Statistics and Computing* (1st ed.). New York: Springer. http://doi.org/10.1017/CBO9781107415324.004

Rahmstorf, S., & Coumou, D. (2011). Increase of extreme events in a warming world. *Proceedings of the National Academy of Sciences*, *108*(44), 17905–17909. http://doi.org/10.1073/pnas.1201491109

Seger, J., & Brockmann, H. J. (1987). What is bet-hedging? *Oxford Surveys in Evolutionary Biology*, *4*, 182–211. http://doi.org/10.1017/CBO9781107415324.004

Team, T. R. C. (2016). *R: A Language and Environment for Statistical Computing*. *R: A Language and Environment for Statistical Computing*. R Foundation for Statistical

Computing. http://doi.org/10.1017/CBO9781107415324.004

Therneau, T. M. (2015). *Package "coxme." Mixed Effects Cox Models*.

Vasseur, D. A., DeLong, J. P., Gilbert, B., Greig, H. S., Harley, C. D. G., McCann, K. S., … O'Connor, M. I. (2014). Increased temperature variation poses a greater risk to species than climate warming Increased temperature variation poses a greater risk to species than climate warming. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, *281*.

Vaswani, M., Linda, F. K., & Ramesh, S. (2003). Role of selective serotonin reuptake inhibitors in psychiatric disorders: a comprehensive review. *Progress in Neuro-Psychopharmacology & Biological Psychiatry*, *27*(1), 85–102. http://doi.org/S0278-5846(02)00338-X [pii]

Visser, M. E. (2008). Keeping up with a warming world; assessing the rate of adaptation to climate change. *Proceedings of the Royal Society of London. Series B, Biological Sciences*, *275*(January), 649–659. http://doi.org/10.1098/rspb.2007.0997

Weinberger II, J., & Klaper, R. (2014). Environmental concentrations of the selective serotonin reuptake inhibitor fluoxetine impact specific behaviors involved in reproduction, feeding and predator avoidance in the fish Pimephales promelas (fathead minnow). *Aquatic Toxicology*, *151*, 77–83. http://doi.org/10.1016/j.aquatox.2013.10.012

Weston, J.J., Huggett, D.B., Rimoldi, J., Foran, C.M., Stattery, M. (2001). Determination of fluoxetine (Prozac^{TM}) and norfluoxetine in the aquatic environment. *Annual Meeting of the Society of Environmental Toxicology and Chemistry*, Baltimore, MD.

Zhang, C., Jansen, M., De Meester, L., & Stoks, R. (2016). Energy storage and fecundity explain deviations from ecological stoichiometry predictions under global warming and size-selective predation. *Journal of Animal Ecology*, *85*, 1431–1441. http://doi.org/10.1111/1365-2656.12531